Facultative to strict anaerobes ratio in the preterm infant microbiota: a target for intervention?

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Abstract

During recent years there has been an increasing interest on the development of strategies for modulating the process of microbiota establishment in preterm infants. For successfully developing such strategies a detailed knowledge of the microbiota establishment process in these infants is needed. In a previous study we evidenced clear alterations in the process of microbiota establishment in preterm newborns when compared with a control group of full-term breast-fed infants. Here we have analyzed these data more in depth, corroborating a reduced proportion of strict anaerobes with respect to facultatives in the fecal microbiota of preterm infants. The potential benefits, as well as the side-effects, of strategies aimed at counterbalancing this alteration in the facultative to strict anaerobes ratio are discussed in this addendum.

Introduction

The basis of a healthy intestinal microbiota lies in early infancy, the initial establishment of the microbiota being a key step for the long term well-being.[1] This establishment starts with facultative anaerobes such as enterobacteria and enterococci and continues with anaerobic genera, including *Bifidobacterium*, *Bacteroides*, and *Clostridium*. The mode of delivery, feeding habits or use of medication, among other factors, may affect this process.[2] These initial stages of microbiota development may be the appropriate time for microbiota modulation towards the establishment of a healthy microbial profile in the individual.

Breast-milk is known to play an important role in the establishment of the intestinal microbiota as well as on the later health of the infant.[3] This has led to the consideration of the fecal microbiota profile of the healthy full-term, vaginally-delivered, exclusively breast-fed (FTVDBF) infant as the standard for a healthy infant microbiota.

Preterm infants, especially those born before week 28, present an immature immune system[4] and a compromised gut mucosa with increased permeability.[5] These represent a risk for infection, at the first moment for vertically transmitted infections, and later on for late-onset nosocomial infections. Nosocomial sepsis in preterm infants is often related to the use of catheters; thus, Gram + microorganisms belonging to the genus *Staphylococcus* are the main causative agent followed by Gram – bacteria, in most cases *E. coli* or Klebsiella,[6,7] these Gram – microorganisms promoting the higher morbidity and mortality rate. In addition, in preterm newborns the process of microbiota establishment is altered, likely due to organ immaturity, the

frequent use of antibiotics and the stay at the Hospital Neonatal Unit. In general, colonization by commensals seems to be delayed and there is an increased colonization by potential pathogenic microorganisms.[8] Premature infants also present an increased risk for developing necrotizing enterocolitis (NEC) and in a significant number of cases these infants suffering NEC develop sepsis, mainly due to enterobacteria, in which the intestinal microbiota is suspected to be the source of infection. Therefore, preterm infants would benefit from intervention strategies directed at favoring the establishment of a healthy microbiota. However, in order to select the best suited intervention strategies to counteract microbiota aberrancies, a detailed knowledge of the intestinal microbiota composition and activity in the target population, such as preterm infants, is needed.[9]

Different studies reported on the administration of pro- and prebiotics to preterm newborns without evidencing adverse events.[10-15] Moreover, some beneficial effects of probiotic and prebiotic administration to preterm neonates have been observed. Recent reviews and meta-analysis have reported benefits of supplementation with some probiotic strains, especially relating to prevention of NEC[16-18] and guidelines for probiotic use in preterm infants have been published.[19] With regard to prebiotics a meta-analysis of randomized controlled trials indicated that prebiotic-supplemented formula increases the levels of bifidobacteria and lactobacilli in premature newborns without adverse effects.[20] However, the current evidence is still insufficient to allow extracting conclusions regarding prebiotics clinical use in preterm babies.[21]

Microbiota establishment in preterm neonates

We have recently studied the process of establishment of the intestinal microbiota in preterm infants (n=21, mean gestational age 32.7 weeks, birthweights ranging from 1190 to 2820 gr) and compared it with that of FTVDBF healthy neonates (n=20, mean gestational age 39.3 weeks, birthweights 3020-4160 gr), by using DGGE, SCFAs analyses and quantitative PCR for eighteen different microbial groups (including, among others, predominant intestinal microorganisms such as *Bacteroides* group, *Clostridium leptum* group, *Blautia coccoides* group, *Lactobacillus* group, *Bifidobacterium*, *Enterobacteriaceae*, *Enterococcaceae* or *Streptococcus*, as well as potentially pathogenic bacteria such as *Klebsiella pneumoniae*, *Weissella*, *Staphylococcus aureus*, *Clostridium perfringens* or *Clostridium difficile*).[22] We observed noticeable quantitative differences in the levels of several microbial populations between both groups of infants during the first three months of life. Preterm infants harbored higher levels of *Enterobacteriaceae, Lactobacillus, Enterococcaceae* and *Weissella*, among others. More specifically these infants, showed increased numbers of *K. pneumoniae*, a relevant nosocomial pathogenic microorganism in this population.[6] On the opposite, preterm babies had lower levels of *Bifidobacterium, Bacteroides* and *Atopobium.* All together these observations seem to indicate a deficiency in the establishment of the normal anaerobic gut microbiota in preterm newborns, as suggested by the increased levels of most facultative anaerobic microorganism, such as *Enterobacteriaceae*, *Enterococcaceae* or *Weissella*, together with the reduced levels of strict anaerobes. Therefore, it could be hypothesized that the differences found between preterm and FTVDBF newborns respond to different gut-environment factors rather than to any specific microbial groups.

In the present addendum we calculated the percentages of each bacterial group with regard to the total number of microorganisms quantified (Figure 1). *Enterobacteriaceae* ranged between 45 to 63% of total microorganisms in FTVDBF infants, whereas they represented 60 to 83% in the preterm group. Among the non-*Enterobacteriaceae* microorganisms, *Bacteroides*, *Enterococcaceae* and *Streptococcus*, followed by *Bifidobacterium* were predominant at 2 days of age in feces from FTVDBF babies. In the preterm group, however, the predominant microorganisms at this time were *Enterococcaceae* and *Lactobacillus* group, followed by *Streptococcus*. Between 10 days and 3 months of age *Bifidobacterium and Bacteroides* predominate in FTVDBF infants, in contrast with the dominance of *Enterococcaceae* and lactobacilli observed in premature babies.

Facultative and strict anaerobes in preterm neonates

Overall we estimated the proportions of facultative and strict anaerobes by the sum of the corresponding microbial groups (*Enterobacteriaceae*, *Enterococcaceae*, lactobacilli group, *Weissella*, *Streptococcus* and *Staphylococcus* for facultatives and *Bifidobacterium*, *Bacteroides* group, *Clostridium* cluster IV, *Clostridium* cluster XIVa and *Atopobium* group for strict anaerobes) and found statistically significant differences between both infant groups during the first three months of life (Figure 2).

To further assess this empirical observation of altered facultative and strict anaerobes levels, we used a mathematical approach, by means of factorial analysis, for grouping the different microbial populations quantified in our previous study.[22] This analysis evaluates the existence of linear relationships among variables (microbial groups), grouping them into “factors” if such

relationships do exist. To this end those microbial groups detected in our previous study at very low frequencies (such as *Clostridium difficile*, *Desulfovibrio*, *Clostridium perfringens*, *Shigella*, *Staphylococcus aureus* or *Akkermansia*) , and therefore not likely being members of the normal gut microbiota of newborns, were excluded from the analysis. The factorial analysis using correlation matrices and Principal Component Analysis as extraction method (SPSS software; SPSS Inc., Chicago, USA), was then applied to the data. Three main factors, showing eigenvalues higher than 1 (3.27, 2.44 and 1.41, respectively) and explaining 25, 19 and 11% of variance, respectively, were obtained. These factors clustered the original variables corresponding to specific microbial groups. For the first factor the variables showing higher coefficients were *Weisella, Enterobacteriaceae*, lactobacilli and *Enterococcaceae*, all of them facultative anaerobic microorganisms. The second factor included *Bifidobacterum*, *Bacteroides*, *Atopobium* and *Clostridium* clusters IV and XIVa, all of them strict anaerobes. This prompted us to define these factors as “facultatives” and “anaerobes”, respectively. Interestingly the third factor grouped staphylococci and streptococci, the numerically predominant microorganisms found in breast milk,[23] thus being named “breast-milk” factor.

The values obtained from each infant’s fecal sample, for these newly created factors, were used for comparing between preterm and FTVDBF newborns at different time points during the first three months of life (2, 10, 30 and 90 days of age). At 2 days of age the three factors showed statistically significant differences between both groups of infants (ANOVA, $p<0.05$), “facultatives” showing a higher value in preterms whilst the contrary was

observed for “anaerobes” and “breast-milk”. At later sampling points only the factors “facultatives” and “anaerobes” differed significantly ($p<0.05$ for both factors at 10, 30 and 90 days) being higher and lower, respectively, in premature infants (data not shown). Thus, these two factors allowed discriminating between both infant populations during the first months of life (Figure 3).

With regard to the factor “breast-milk” the difference observed at 2 days, but not at later sampling points, could be probably related with the feeding habit of the infants. All infants in the control group (FTVDBF) were exclusively breast-fed during the whole duration of the study, whilst none of the preterm infants was exclusively in breast-feeding. In our cohort at 2 days of age only 2 preterm infants were exclusively breast-fed, 3 were on mixed feeding and 16 were on formula, whilst at 10 days only 3 infants remained exclusively formula-fed. These differences in feeding habits are very likely the explanation for the differences obtained for the factor “breast-milk” at 2 days of age.

The factorial analyses of microbial groups in FTVDBF and premature babies commented just above appears to support our initial empirical observation of reduced levels of strict anaerobic microorganisms and increased facultatives in preterm infants. Therefore, it can be speculated that the differences between both groups of infants may be based on an altered oxidative environment in the gut rather than on differences on specific microorganisms, although other factors may be involved as well. Noteworthy, in an animal model it has been shown by other authors that the environmental parameter having the strongest influence on the intestinal bacterial community was the redox potential.[24]

On this basis it can be hypothesized that nutritional interventions aimed at increasing strict anaerobes and reducing the levels of facultative anaerobic microorganisms, which include several potentially pathogenic bacteria, may be beneficial for preterm infants.

Intestinal oxidative stress as a potential target for microbiota modulation in preterm neonates

At birth the neonate must adapt from the hypoxic fetal environment to the relatively hyperoxic atmospheric environment, which causes an important oxidative stress. Moreover, very often preterm infants are oxygenated with high concentrations of O_2. Recent studies have demonstrated that premature birth is associated with an increased oxidative stress, and damage promoted by reactive oxygen species that are not properly managed by the immature antioxidant systems of preterm babies.[25-28] This increased level of oxidative stress has been related to the risk for the so called “free radical-related diseases”, such as retinopathy, bronchopulmonary dysplasia and, at intestinal level, NEC[29] which are often associated with preterm birth. Interestingly, breast-milk from mothers of full-term infants has been found to have a higher antioxidant capacity than that of mothers of preterm babies.[30] These findings have prompted researchers to focus their attention on antioxidant supplementations in preterm babies by the use of non-enzymatic proteins, such as transferrin and ferritin, antioxidant enzymes or oxidizable molecules, including vitamins, fatty acids or aminoacids, among others,[26,27] for some of which positive results have been published.[31,32] Interestingly, probiotic microorganism could also play a role as antioxidants, since strains displaying this property have been reported[33] and probiotic metabolites able to prevent the

production of proinflammatory cytokines induced by oxidative stress have been recently identified.[34]

Therefore, in the context of nutritional interventions the inclusion on infant formula of antioxidants, able to reduce the redox potential also in the intestine, represents a hypothetical, but nevertheless attractive, approach for microbiota modulation in preterm infants. Despite the potential beneficial effects of such interventions on terms of microbiota composition, by reducing the levels of facultative anaerobes which include the most relevant nosocomial infectious agents for this infant population,[6,7] no attention has ever been paid to the effect of such interventions on intestinal microbiota in these infants. However, in addition to the potential benefits of such strategy there are also some potentially deleterious effects that should be carefully evaluated. Among these the risk of bowel isquemia should be considered. A study that, although controversial, underlines this point is that of Besselink et al.[35] in which the use of enterally administered probiotics to reduce pathogen overgrowth in patients with severe acute pancreatitis was studied. The authors did not find significant differences between groups in infectious complications or in new onset organ failure, but the test group doubled the mortality rate as compared with the placebo group, and bowel ischemia was detected during surgery or autopsy in nine patients in the probiotics group and none in the placebo group. The authors suggested that enteral administration of probiotics further increased local oxygen demand, with a combined deleterious effect on an already critically reduced blood flow. Although, these results should be taken with caution[36] this study highlights the need of a careful safety evaluation of any intervention in highly susceptible populations.

Current and future challenges

In preterm infants the main benefits of microbiota modulation would include the enhanced maturation of the immune system and the reduction in the number of potentially pathogenic microorganisms that may cause infection in this highly susceptible population. Our results underline the global differences occurring on the process of establishment of the intestinal microbiota in preterm infants when compared with the healthy standard population (FTVDBF infant). This may be of help for designing new intervention strategies targeting the gut microbiota in order to minimize the risk of infection and/or NEC in these infants. However, it must be pointed out that more research is needed to assess whether that of FTVDBF infants represents a good microbiota model also for preterm infants. In addition our results did not include data on extreme-preterm-infants, which would be the group that could benefit the most from microbiota modulation, but for which we do not really know whether the same differences do exist.

It is worth mentioning that, similarly as for preterm neonates and NEC where both oxidative stress[29] and microbiota factors[37,38] seem to play a role, inflammatory bowel disease is another situation in which the microbiota has been found to be altered[39] and for which a role of oxidative stress as patho-physiological factor has been reported.[40] This suggests that oxidative stress may be an important factor driving the microbiota composition in different inflammatory diseases.

In this addendum we look at our previous data from a different perspective and tried to understand what the global microbiota composition of preterm infants may tell us in terms of environmental conditions. This allowed us to

speculate that oxidative stress may be an important factor shaping the gut microbiota in these infants and, therefore, it also represents a potential target for intervention. This hypothesis is just speculative and should now be experimentally tested. To this regard, we suggest to consider the microbiota among the potential beneficial targets in intervention studies aiming at reducing the oxidative stress in preterm infants.

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All authors declare no conflict of interest.

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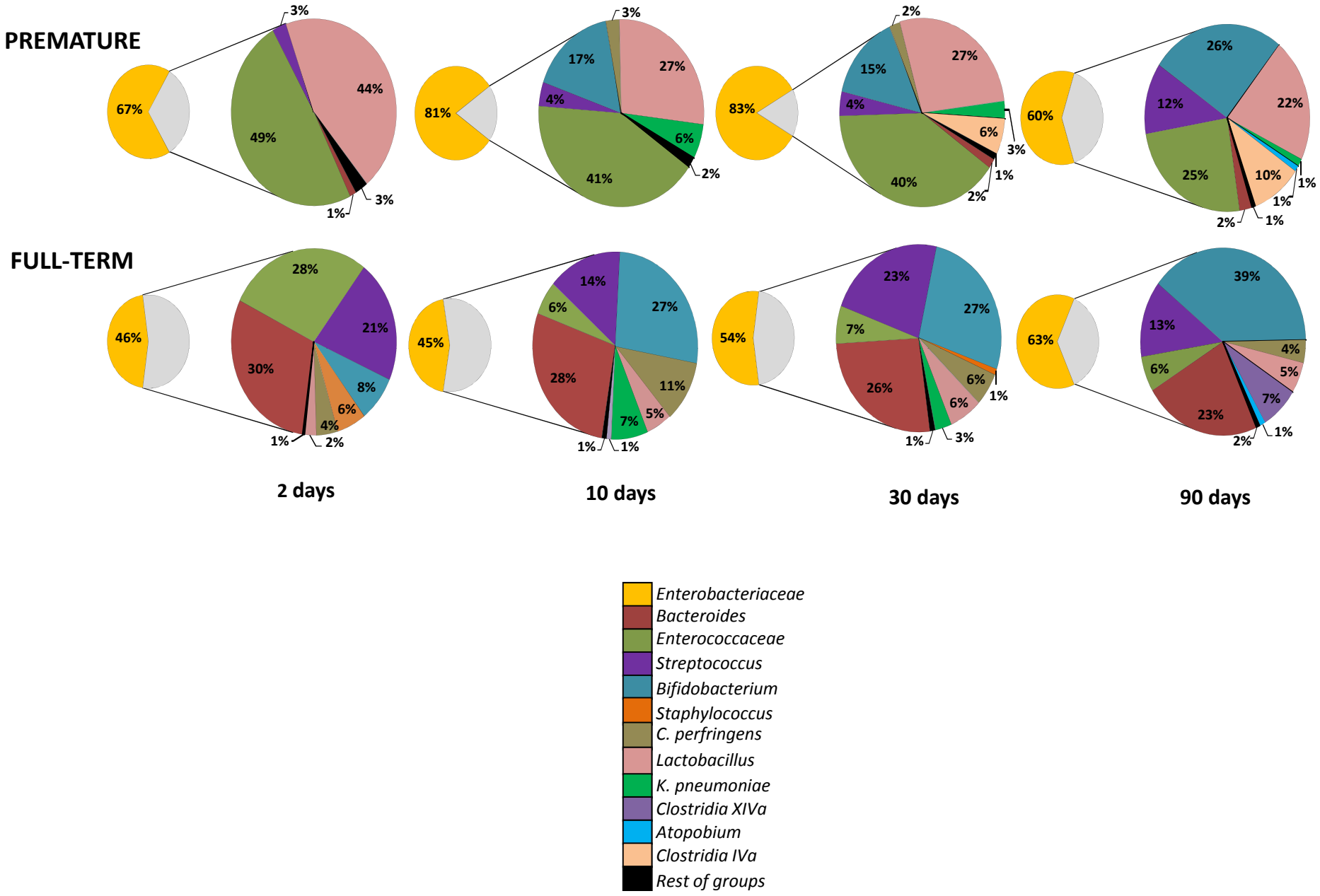
Figure 1. Percentages of different fecal microbial groups, in both groups of infants, at 2, 10, 30 and 90 days of age.

Figure 2. Percentages of facultative anaerobes and strict anaerobes in premature and full-term infants at the different time points analyzed. Statistical comparison (U Mann-Whitney) between both groups.

Figure 3. Scatter-gram of the infant samples at the different sampling points according to the values obtained for the factors “facultatives” and “anaerobes”. Open circles: Full-term, vaginaly-delivered, exclusively breast-fed babies. Close circles: Preterm babies.


PREMATURE
FULL-TERM
67%
3%
44%
49%
1%
3%
81%
3%
17%
27%
4%
6%
41%
2%
83%
2%
15%
27%
4%
6%
3%
40%
2%
1%
60%
26%
12%
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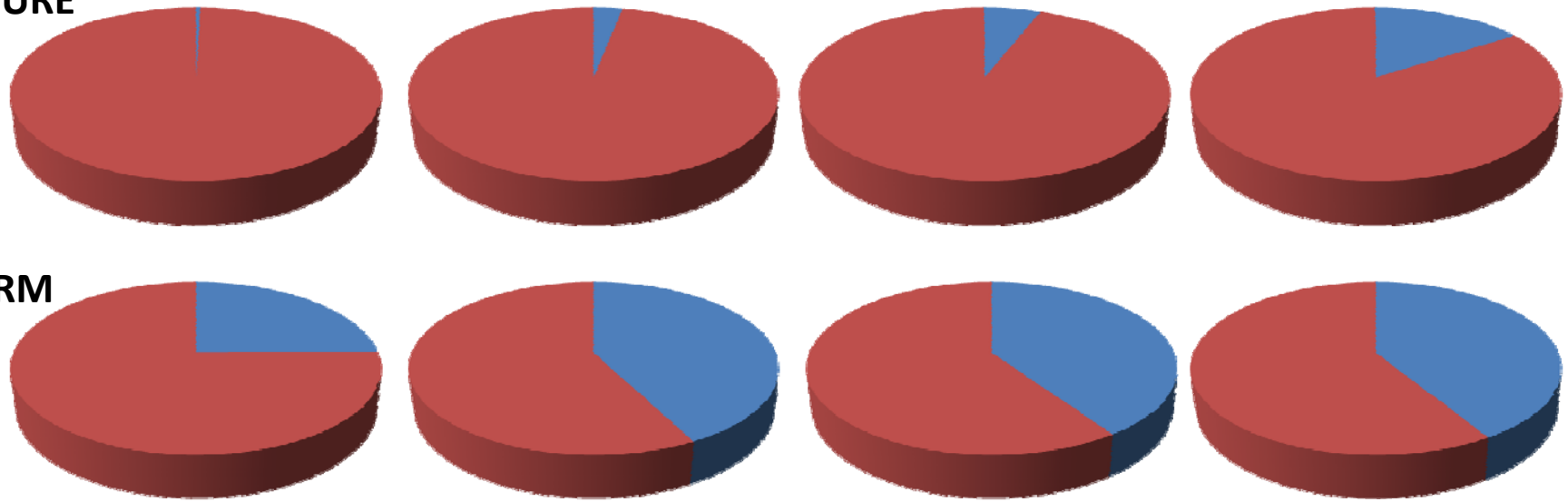
2 days
10 days
30 days
90 days
Enterobacteriaceae
Bacteroides
Enterococcaceae
Streptococcus
Bifidobacterium
Staphylococcus
C. perfringens
Lactobacillus
K. pneumoniae
Clostridia XIVa
Atopobium
Clostridia IVa
Rest of groups

PREMATURE

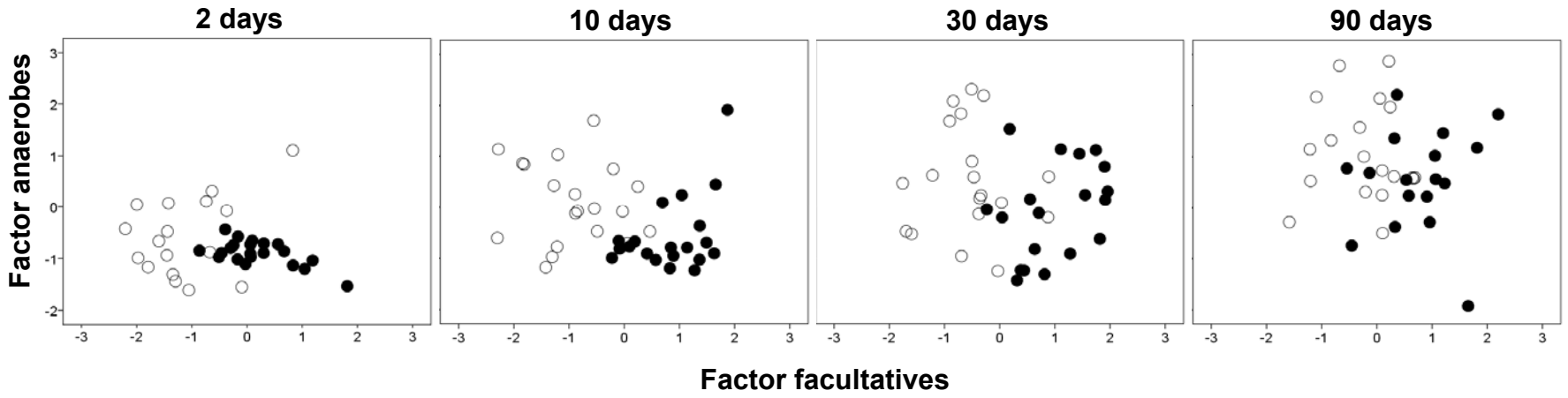
FULL-TERM



2 days	10 days	30 days	90 days
p.value 0.007	*p.value < 0.001*	*p.value 0.002*	*p.value 0.048*

Facultative anaerobes

Strict anaerobes


2 days
10 days
30 days
90 days
Factor anaerobes
Factor facultatives